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A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   27 April 2011

Thank you for the submission of your manuscript "A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer". We have now heard back from the two referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise some concerns on the study, which should be addressed in a revision of the manuscript.

In particular, Reviewer #1 notes that numerical values should be used in all cases when the performance of the diagnostic test is investigated. Of note, both Reviewers highlight that the manuscript would benefit from a discussion of the known functions of the selected miRNAs.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

-The authors submit a methodical and well-described manuscript for serum miRNA biomarkers to identify high-risk asymptomatic individuals with early-stage NSCLC.

-There are some areas that could use grammar corrections/improvement. Example: Simpler is not an English word. Simple methodologies is correct.

-It not clear to when reviewing if the 34 miRNA signature was assessed moving forward in the testing and subsequenct sera samples or were 147 miRNAs measured in all these additional samples?

-Can the authors explain why 34 miRNAs were required for a classifier on a training set of n=64 samples. Each individual miRNA is probably not contributing much. How can they ensure that there is not overfitting bias? As they point out, Boeiri et al use only 13 miRNAs to classify. Can the authors show a graph of the relative change in AUC for their PCR results as there an increasing number of biomarkers selected?

-Can the authors relate/describe how any of their selected miRNAs may be involved in lung carcinogenesis citing current literature? It would add clinical/translational value to the paper.

-Can the authors report their results in the text with numerical values rather than stating "performed remarkably well" and referring to a figure?
"When the 34-miRNA predictor was applied to evaluate the risk in the symptomatic set and in the PH set, it performed remarkably well (Fig. 2C). The average risk index of NSCLCs patients was virtually indistinguishable, both for ACs and SCCs, between the symptomatic set and the testing set of the COSMOS trial."

And

"In addition, the average risk index of patients from the symptomatic set was clearly higher, in a statistically significant manner, than that of PH-harboring patients (Fig. 2C, right)."

-Difficult to follow, clearly state what would be the expected false positive rate if this assay were applied to patients that fit the eligibility criteria for the COSMOS trial "There were no significant differences in the average risk of the normal and nodule categories in spite of the fact that the 34-miRNA model and the risk algorithm were derived by training on a dataset (the training set) that did not include nodules (Fig. 3A)."

-Is the take away message of this statement that the classifier will not detect high risk for careful follow-up but only those that have NSCLC that can be visualized on imaging? "When the risk predictor algorithm was applied, it indicated a significantly increased average risk index for sera collected after the onset of the disease (average risk BDO,-7.1; Tumor, 10.4; $p < 0.001$, paired t-test; Fig. 3B). Thus, at least in the cases analyzed, the 34-miRNA model was capable of detecting the conversion from a normal to a malignant state."

-Support this statement with a reference. How much would assay cost in a certified lab or with assay in pathology department with stringent positive/negative controls or even point of care test? "2) it is considerably cheaper, easier and more immediately implementable (particularly from the point of view of patient accrual and compliance) than current screening procedures"

-Can differences between this study and (Boeri et al, 2011), be in part due to different analyte, serum vs plasma?

Referee #2:

The authors in this manuscript reported that they developed a test for early stage NSCLC based on the detection of 34 miRNAs from serum. They showed that the test could identify early stage NSCLC patients in a population of asymptomatic high-risk individuals with 80% accuracy. The study recruited 59 patients whose pre-operative sera were available from the COSMOS study, in which 5203 high-risk individuals were screened by annual LD-CT to detect lung cancer. The patients were assigned to training set and testing set. A multivariate risk predictor using the weighted linear combination of the 34 miRNA expression values was developed to detect asymptomatic NSCLC. The authors claimed that the signature may be useful to distinguish between benign and malignant lesions, and to capture the onset of the malignant disease in individual patients over time.

The study is intriguing and the signature may have potential for clinical application is the signature can be verified in independent and prospective larger cohort. They are several comments which may be useful to improve the manuscript.

1.In the independent validation of signature, the authors used the pre-operative sera from an independent cohort of symptomatic 26 NSCLC patients (23 AC and 13 SCC), who underwent surgery at the European Institute of Oncology. The sample size of NSCLC patients is relatively small. It will be more convincing the sera markers can be validated in larger independent cohort of symptomatic and asymptomatic NSCLC patients.

2.In the validation for benign lesion, only 15 benign pulmonary hamartomas were included. Again, this may be not robust enough, other benign pulmonary lesions, such as granuloma and chronic inflammatory lesions should also be tested to provide more convincing support for the specificity of the signature.

3.The mechanisms and potential pathways involved in NSCLC progression in this 34 miRNA should be discussed in the discussion section.

4.The manuscript may need proof-read again, particularly the references, some of them are incomplete.

1st Revision - authors' response   14 May 2011

Referee #1:

There are some areas that could use grammar corrections/improvement. Example: Simpler is not an English word. Simple methodologies is correct.

R: Agree. The text has been edited

It not clear to when reviewing if the 34 miRNA signature was assessed moving forward in the testing and subsequent sera samples or were 147 miRNAs measured in all these additional samples?

R: The 147 miRNAs were analyzed in all sera in the study. This is now explained unambiguously in the manuscript, page 5, line 15-17. Of course the 34 miRNA signature was derived only on the data of the training set and applied blindly to all other datasets.

Can the authors explain why 34 miRNAs were required for a classifier on a training set of n=64 samples. Each individual miRNA is probably not contributing much. How can they ensure that there is not overfitting bias?
and
As they point out, Boeri et al use only 13 miRNAs to classify. Can the authors show a graph of the relative change in AUC for their PCR results as there an increasing number of biomarkers selected?

R: Agree. In particular:

1) On the issue of overfitting. We can exclude that there is overfitting. The method we used to select miRNAs is the Diagonal Linear Discriminant Analysis (DLDA) with cross-validation procedure, which is widely recognized as a strong and reliable method to identify multivariate diagnostic models (Pitfalls in the analysis of DNA microarray data: Class prediction methods, Journal of the National Cancer Institute 95:14-18, 2003; Prediction error estimation: a comparison of resampling methods, Bioinformatics 21:3301-3307, 2005).

The method is based on a cross-validation procedure (k-5 fold cross-validation) that is applied to the training. The method consists in subdividing the training set in 5 equal parts (i.e. the K subsets), then one of the k subsets is omitted and the classifier model developed from scratch using a training set consisting of samples in the union of the other K-1 subsets. The performance of the classifier is next evaluated in the omitted subset. The process is repeated 5 times in order to classify all the samples present in each k subset. In addition, to ensure that the estimate is stable we repeated the initial random partitioning (5 subsets) 100 times in order to avoid any possible bias for subset composition. Overall the process of miRNA selection is repeated 500 times by using different combinations of samples, and the final number of miRNAs in the classifier depends on the combination of all the classifiers built during the iterated process. This strategy guarantees that the selection of miRNAs used in the classifier it is not dependent on any particular combination of sample, which ensures that there is no data overfitting, and that – at the same time – the performance of the classifier (accuracy, sensitivity and specificity) is estimated independently of any particular omitted subset.

Even more importantly, overfitting is excluded by the use of independent validations sets, to which the predictor is applied blindly. When applied to the testing (validation) set from the COSMOS study, for instance, the predictor displayed acc. 80%, sens. 71%, and spec. 90%. In addition, the values in the testing set are remarkably similar to those of the training set (acc. 78%, sens. 69% and spec. 84%), providing further support to the notion that there is no data overfitting.

2) On the issue of the signature by Boeri *et al.* We can only speculate about why the Boeri classifier is composed of a lower number of miRNAs. This issue is now discussed in the manuscript in a more extended fashion (see page 10, lines 14-25 page 11 lines 1-10). One possibility is that they used a different strategy to select miRNAs (i.e. without using cross-validation or other similar statistical methods to determine whether the classifier predicted accurately). Unfortunately, we cannot compare the data and the methodology directly, since they do not describe the exact procedure used to build their classifier.

3) On the issue of the contribution of each miRNA to our predictor. The reviewer is right, some miRNAs have a greater impact, and others a comparatively minor one. However, the reported composition is the best possible one, under our conditions of analysis. The reviewer's suggestion to show the performance of the predictor using progressively increasing number of miRNAs is a good one, and we have added this information to the manuscript. As we now show in Figure S5, a "core" 5-miRNA signature is already able to produce an AUC of 0.77 in the testing set. Progressively increasing the number of miRNAs, improves the results up to an AUC of 0.89 with the 34-miRNA model.

Can the authors relate/describe how any of their selected miRNAs may be involved in lung carcinogenesis citing current literature? It would add clinical/translational value to the paper.

R: Agree. This is now described on page 11, lines 13-25 and page 12, lines 1-11.

Can the authors report their results in the text with numerical values rather than stating "performed remarkably well" and referring to a figure? "When the 34-miRNA predictor was applied to evaluate the risk in the symptomatic set and in the PH set, it performed remarkably well (Fig. 2C). The average risk index of NSCLCs patients was virtually indistinguishable, both for ACs and SCCs, between the symptomatic set and the testing set of the COSMOS trial." And "In addition, the average risk index of patients from the symptomatic set was clearly higher, in a statistically significant manner, than that of PH-harboring patients (Fig. 2C, right)."

R: Agree. Numerical values have been added to the main text in all instances.

Difficult to follow, clearly state what would be the expected false positive rate if this assay were applied to patients that fit the eligibility criteria for the COSMOS trial "There were no significant differences in the average risk of the normal and nodule categories in spite of the fact that the 34-

miRNA model and the risk algorithm were derived by training on a dataset (the training set) that did not include nodules (Fig. 3A)."

R: Agree. The text has been modified to comply with the reviewer's request (page 8, lines 23-25 and page 9, lines 1-2).

Is the take away message of this statement that the classifier will not detect high risk for careful follow-up but only those that have NSCLC that can be visualized on imaging? "When the risk predictor algorithm was applied, it indicated a significantly increased average risk index for sera collected after the onset of the disease (average risk BDO,-7.1; Tumor, 10.4; p <0.001, paired t-test; Fig. 3B). Thus, at least in the cases analyzed, the 34-miRNA model was capable of detecting the conversion from a normal to a malignant state."

R: The reviewer is right. At the present state of knowledge, our data only allow to say that, in the limited population tested (for which BDO sera were available), our predictor shows the same level of accuracy as LD-CT (i.e. allows for detection of seroconversion, which correlates with the appearance of lesions by LD-CT). It is possible that the predictor (or a subset of it) can be developed into a "pre-diagnostic signature", i.e. a signature capable of detecting the risk of developing cancer (or of developing LD-CT-detectable lesions before they appear). Presently, this is something that we are investigating, by collecting samples from the COSMOS volunteers over time. This will require some time and effort, since we have to collect samples from the entire COSMOS population, over the years, wait for a sufficient number of events to develop (we will need at least 50-60 events for a training cohort and 50-60 for the testing cohort), and then perform the analysis.

Support this statement with a reference. How much would assay cost in a certified lab or with assay in pathology department with stringent positive/negative controls or even point of care test? "2) it is considerably cheaper, easier and more immediately implementable (particularly from the point of view of patient accrual and compliance) than current screening procedures"

R: We did not find specific literature to support our argument. We think, however, that the arguments in favor of a blood test are self-evident.
1. From the financial point of view. We calculate that the test will cost about 50-100 Euros, based on current technologies and platform. This is in agreement with costs of Real Time PCR based diagnostic tests commercialized by molecular diagnostic companies (e.g. http://eng.bioneer.com/; and http://www.kbioscience.co.uk/). We estimate that test analysis will cost ~15 Euros) . Thus, the test will cost approximately 5-10 fold less than a CT scan. We speculate that further development of miRNA quantitation in the blood by others and by us will surely lead to lower costs.
2. From the patient point of view: it avoids unnecessary exposure to X-rays
3. From the accrual point of view: it avoids "medicalization" of asymptomatic individuals; it does not require patients to check in into a hospital.

Can differences between this study and (Boeri et al, 2011), be in part due to different analyte, serum vs plasma?

R: Agree. This possibility is now clearly mentioned (and relevant support literature cited) in the revised Discussion (page 11, lines 1-3).

Referee #2:

1. In the independent validation of signature, the authors used the pre-operative sera from an independent cohort of symptomatic 26 NSCLC patients (23 AC and 13 SCC), who underwent surgery at the European Institute of Oncology. The sample size of NSCLC patients is relatively small. It will be more convincing the sera markers can be validated in larger independent cohort of symptomatic and asymptomatic NSCLC patients.

R: We respectfully point out that we have used 2 independent validation cohorts, and not 1, as stated by the reviewer. The first cohort was from asymptomatic individuals (the testing cohort) and comprised 30 Normal, 33 Nodules, 22 AC and 12 SCC; the second was from symptomatic patients (the one referred to by the reviewer), containing 23 AC, 13 SCC and 15 PH). Our predictor was therefore validated on a total of 148 samples (30 Normal, 33 Nodules, 45 AC, 25 SCC and 15 PH).

Thus, while the need for a larger validation study remains (as also clearly pointed out in our Discussion, page 12, lines 14-18, the validations reported in the present manuscript are by no means performed on a "small cohort".

2. *In the validation for benign lesion, only 15 benign pulmonary hamartomas were included. Again, this may be not robust enough, other benign pulmonary lesions, such as granuloma and chronic inflammatory lesions should also be tested to provide more convincing support for the specificity of the signature.*

R: Again, we respectfully point out that in our validation for benign lesions, we analyzed 33 nodules (from asymptomatic patients) and 15 PH (from symptomatic patients. While larger studies are undoubtedly required (see previous point), both in the case of nodules and PH, the specificity of the signature was rather convincing. We would like to add that, although we do not know the histology of the 33 CT detected nodules that we screened (patients were not operated), these nodules with benign behavior at subsequent follow-up CT scans include with high probability both pulmonary granulomas and chronic inflammatory lesions.

3. *The mechanisms and potential pathways involved in NSCLC progression in this 34 miRNA should be discussed in the discussion section.*

R: Agree. This is now described on page 11, lines 13-25 and page 12, lines 1-11.

4. *The manuscript may need proof-read again, particularly the references, some of them are incomplete.*

R: Agree. The text has been edited

Additional correspondence   30 May 2011

Thank you again for your submission to EMBO Molecular Medicine. We have now received the report from the referee who was asked to re-assess the manuscript. The report is copied below. I would like you to incorporate the change detailed below before we can proceed with the official acceptance of your manuscript.

As you will see, the referee acknowledges that the manuscript was significantly improved during revision. However, he/she raises an issue that should be addressed. We would strongly encourage you either provide the requested information or to tone down the corresponding statement.

The statement could be amended the following way:
2) it is likely going to be considerably cheaper, easier.....

I look forward to your answer via reply e-mail.

Kind regards,

Editor
EMBO Molecular Medicine

Referee#1:

The authors addresses adequately nearly all queries. While they addressed this query below with the following response, it would be appreciated (to set an example for future mirna "diagnostic" work) and add about the potential cost estimate of the assay vs. the proposed current screening paradigm (in NSCLC, low dose CT screening).
>>Support this statement with a reference. How much would assay cost in a certified lab or with assay in pathology department with stringent positive/negative

controls or even point of care test? "2) it is considerably cheaper, easier and more immediately implementable (particularly from the point of view of patient accrual and compliance) than current screening procedures"

R: We did not find specific literature to support our argument. We think, however, that the arguments in favor of a blood test are self-evident.

1. From the financial point of view. We calculate that the test will cost about 50-100 Euros, based on current technologies and platform. This is in agreement with costs of Real Time PCR based diagnostic tests commercialized by molecular diagnostic companies (e.g. http://eng.bioneer.com/; and http://www.kbioscience.co.uk/). We estimate that test analysis will cost ∼15 Euros) .

Thus, the test will cost approximately 5-10 fold less than a CT scan. We speculate that further development of miRNA quantitation in the blood by others and by us will surely lead to lower costs.

2. From the patient point of view: it avoids unnecessary exposure to X-rays

3. From the accrual point of view: it avoids "medicalization" of asymptomatic individuals; it does not require patients to check in into a hospital.

Additional correspondence   31 May 2011

Thank you very much for your mail of May 30th 2011, concerning our manuscript "A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer" (EMM-2011-00669-V2).

We have amended the text, to tone down the statement concerning the cost/feasibility of the test, as suggested by you and the reviewer. The statement now reads "it is likely going to be considerably cheaper, easier..." (page 9, line 25 and page 10 line 1).

We hope that the manuscript is now acceptable in EMM.

2nd Editorial Decision   31 May 2011

Please find enclosed the final report on your manuscript. We are pleased to inform you that your manuscript is accepted for publication and will be sent to our publisher to be included in the next available issue of EMBO Molecular Medicine if or once we have received your licenses (see below).

Please see below for additional IMPORTANT information and instructions regarding your article, its publication, and the production process.

Congratulations on your interesting work.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Other Remarks):

The authors addresses adequately nearly all queries. While they addressed this query below with the following response, it would be appreciated (to set an example for future mirna "diagnostic" work) and add about the potential cost estimate of the assay vs. the proposed current screening paradigm (in NSCLC, low dose CT screening).

>>Support this statement with a reference. How much would assay cost in a certified lab or with assay in pathology department with stringent positive/negative controls or even point of care test? "2) it is considerably cheaper, easier and more immediately implementable (particularly from the point of view of patient accrual and compliance) than current screening procedures"

R: We did not find specific literature to support our argument. We think, however, that the arguments in favor of a blood test are self-evident.

1. From the financial point of view. We calculate that the test will cost about 50-100 Euros, based on current technologies and platform. This is in agreement with costs of Real Time PCR based diagnostic tests commercialized by molecular diagnostic companies (e.g. http://eng.bioneer.com/; and http://www.kbioscience.co.uk/). We estimate that test analysis will cost ～15 Euros) .

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